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While the significance of the serine/threonine protein kinase AKT expression and/or activity in human breast cancer has become increasingly evident, consistent alterations of a specific isoform have not been well documented. A specific isoform of AKT may be preferentially activated or activated proteins may have different substrate preferences, providing a therapeutic opportunity to target a particular isoform. The primary endpoint is to compare responsiveness to tamoxifen in ER-α-positive, ErbB2 low tumors with high AKT1 activity versus no AKT1 activity. 7 tumors each of 1) ER-α-positive, ErbB2 high; 2) ER-α-positive, ERbB2 low; 3) ER-α-positive, no ErbB2; 4) ER-negative, ErbB2 high; 5) ER-negative, ErbB2 low; and 6) ER-negative, no ErbB2 will be tested for AKT (AKT1, AKT2, AKT3), ErbB (EGFR, ErbB2, ErbB3, and ErbB4) and ER-α expression and activity. We have received 26 tumors and their surrounding normal tissue. Paraffin sections were prepared from most of these tissues and the sections were immunostained with total anti-Akt, phosphospecific anti-Akt, anti-progesterone receptor, total and phosphospecific antibody against ErbB2. We are in the process of scoring of these paraffin sections. In addition, we prepared cell lysates and RNA from the tumors and normal tissues for further analyses.

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INTRODUCTION

The significance of AKT expression and/or activity in human cancer has become increasingly evident. However, consistent alterations in overexpression and/or activity of a specific AKT isoform in human breast tumors have not been well documented. A specific isoform of AKT may be preferentially activated (1-5) or activated proteins may have different substrate preferences, providing a therapeutic opportunity to target a particular isoform. Our *in vitro* data show that the growth factors EGF, IGF-I, and HRG-β1, as well as estradiol, can activate the PI3/K/Akt pathway in ER-positive breast cancer cells. The effects of estradiol and HRG-β1 are mediated by membrane ER-α and by the ErbB2 but not by the EGFR signaling pathway. Moreover, estradiol and growth factors can induce anchorage-dependent and independent proliferation and membrane ruffling that can be inhibited by antiestrogens, selective ErbB2 inhibitors and by either PI 3-K inhibitors or dominant negative Akt mutants. In contrast, Akt exerts estrogen-like effects on cell growth, membrane ruffling, and ER-α regulation and tamoxifen cannot fully abolish its effect. Taken together, these data suggest that estradiol, EGF, or HRG-β1 interact with membrane ER-α and a heterodimer with ErbB2, leading to tyrosine phosphorylation. This results in activation of the PI 3-K and AKT1. AKT1, in turn, may interact with nuclear ER-α, altering its expression and activity, as well as cell proliferation and response to tamoxifen. Therefore, this proposal will determine ErbB (EGFR, ErbB2, ErbB3, and ErbB4), AKT (AKT1, AKT2, and AKT3), and ER-α expression and activity in 42 breast tumors, their surrounding normal tissue, and stroma. The primary endpoint is to compare responsiveness to tamoxifen in ER-positive, ErbB2 normal (0.1-0.19 pg/cell) tumors with high AKT1 activity versus no AKT1 activity.

BODY:

Task 1: **Selection of frozen tumors (including adjacent control tissue) and paraffin sections)**

Since the Tissue Shared Resource of the Lombardi Cancer Center could not provide us with more that 4 frozen tumor tissues, we contacted the National Disease Research Interchange (NDRI) and we are still in the process of selection of frozen tumors (including adjacent control tissue) and paraffin sections for gathering the 7 samples in each of the following 6 groups of tumors based on ER-α and ErbB2 levels:

Group 1: ER-positive, ErbB2 normal (0.1-0.19 pg/cell)
Group 2: ER-positive, ErbB2 overexpressed (>0.2 pg/cell)
Group 3: ER-positive, ErbB2 negative (<0.09 pg/cell)
Group 4: ER-negative, ErbB2 normal
Group 5: ER-negative, ErbB2 overexpressed
Group 6: ER-negative, ErbB2 negative

We are also requesting follow-up information (tamoxifen responsiveness) as well as traditional prognostic markers (tumor stage, tumor type, nodal status, differentiation, etc.) (See attached Table with Tumor Characteristics).

So far, we have received 26 frozen tumors and 26 adjacent normal tissues and 18 paraffin sections of tumors and 18 paraffin sections of normal adjacent tissue.

Task 2: **Measurement of the expression of ErbB receptors**

a) Lysates from 26 frozen tumors and 26 adjacent normal tissue were prepared
b) Protein expression for ErbB2 was analyzed in sections from 8 tumors and 8 adjacent normal tissue using immunocytochemistry and anti-ErbB2 antibodies (see results in Table 2). The rest of 10 paraffin sections were sent to the Histopathology and Tissue Shared Resources of the LCC for slide preparation and immunostaining.
In addition, we are testing EGFR, ErbB3, and ErbB4 antibodies for Western blot as well as immunohistochemistry, as well as phosphospecific antibodies to determine the activity of ErbBs in all the tumors.

Task 3: **Determination of the expression of AKT1, AKT2, AKT3 in frozen tumor tissue**

a) We designed the primers for RT-PCR and ordered them
b) We are currently testing the primers by RT-PCR
c) We detected protein expression for total Akt by immunostaining of paraffin sections of 8 tumors with total anti-Akt antibody. Scores as intensity of staining were assigned - this reflects level of expresssion low (1+) to high (3+). Also percent of tumour positivity (entire, partial or localized) were noted.

Task 4: **Measurement of the activity of Akt**

a) Immunostaining of paraffin sections of 8 tumors and their adjacent normal tissues was performed using phosphospecific anti-Akt (P-Akt) antibody to detect whether activation of Akt is derived from tumor cells or stromal tissue.

KEY RESEARCH ACCOMPLISHMENTS, REPORTABLE OUTCOMES, and CONCLUSIONS

- We are still selecting tissues for the 6 tumor groups. So far we have 4 tumors in group 6, 3 tumors in group 4, 2 tumors in group 3, and 1 tumor in group 2. We expect to collect all tumors by the summer of 2005

We are still gathering follow-up information about: age of the patient, tumor histology, ER, PR, ErbB2 status of the tumor, as well as tamoxifen response.

- We prepared lysates from 26 frozen tumor and 26 adjacent normal tissue samples and are in the process of analyzing ErbB receptors, AKT isoforms expression and activity and AKT isoform mRNA
- We have analyzed 8 tumors and 8 adjacent normal tissues by immunohistochemistry for ErbB2, PR, Akt and phosphospecific Akt (Akt activity) for localization and staining intensity
- A very good correlation was observed between the ErbB2 and progesterone receptor PgR immunohistochemical score that was obtained from NDRI and the measured score in the sample of 8 tumors

- Akt staining occurs in ductal areas of tumors and in cell groups surrounded by lymphocytes and inflammatory cells. Its expression is higher in the cytoplasm than in the nucleus. Adjacent normal tissue can also express Akt, but the staining intensity is lower
- Akt activity is usually correlated with Akt expression in both tumor and normal surrounding tissue. In normal tissue, hyperplastic ducts and scattered end units are also positive for active Akt. Nuclear staining is higher for active Akt when compared to expressed Akt.

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APPENDICES:

1) Table 1: Tumor Characteristics

2) Table 2: Immunohistochemical Analysis of 8 Tumors (CA) and Matched Adjacent Normal Tissue (N or NAJ) for Akt, P-Akt, PgR, and ErbB2

Tumor Characteristics

#	Tumor Type	Lymph Nodes	Age	ER	PR	ErbB2	Other
1	invasive ductal (comedo) carcinoma grade3	0/12	88	-	-	-	
2	ductal carcinoma, poorly differentiated gr.3	0/15	51	-	-	-	
3.	ductal carcinoma, poorly differentiated gr.2-3	0/6	61	-	-	1	
4.	bilateral infiltrating duct carcinoma, grade 3	1metastatic	52	-	-	2	
5.	invasive ductal carcinoma, grade 3 (nuclear gr.2)	-	50	-	-	-	
6.	mucinous adenocarcinoma, grade 2	0/5	84	+	+	-	
7.	invasive ductal carcinoma, grade 2 (nuclear gr. 3)	4/7	38				
8.	infiltrating ductal carcinoma, grade 3, poorly diff.	ND	87	+	-	3	
9.	infiltrating ductal carcinoma, grade 3	5/9	57				
10.	invasive carc., metaplastic, squamous cell, gr.2	0/5	69				
11.	ductal carc., poorly diff.with dormal lymph. inv. (rec)	ND	72				
12.	infiltr. duct. adenocarc.with lympha & pagetoid, gr2	-	73	+	+	-	
13.	high gr. (nucl.3) in situ ductal carc. w 2 foci of microinv.	0/5	39				
14.	invasive ductal carcinoma, gr.2	0/9	78				
15.	residual intraductal carcinoma	2/8	62				
16.	invasive ductal carc. in fibromuscular tissue, gr.2-3	ND	69				
17.	ductal carcinoma						
18.	invasive lobular carcinoma, gr. 2	1/2	77				
19.	invasive ductal carc., gr.3	ND	88				
20.	invasive ductal carcinoma, gr.2	0/17	74				
21.	spindle cell sarcoma, intermed.-high grade	1/11	69	-	-	-	
22.	infiltrating carcinoma, gr. 3	0/8	50	-	-	2	
23.	infiltrating ductal carcinoma, gr. 2	0/3	58				
24.			80				
25.	invasive ductal carc. w medullary features, gr. 3	0/4	81				
26.	infiltrating ductal invasive carc., gr. 2	17/17	80				

Table 2: Immunohistochemical Analysis of 8 Tumors (CA) and Matched Adjacent Normal Tissue (N or NAJ) for Akt, P-Akt, PgR, and ErbB2

#	Specimen ID	ID #	Description	Akt	pAkt	PgR	ErbB2
1.	12746-05	1390 E	L Breast CA	1. ductal areas and solid groups surrounded by lymphocytes and inflammatory cells stained 1-2+ cytoplasm. No nuclear staining. 2.Undifferentitiated invasive tumor in small groups were very weak or negative. 3. Undifferentiated infiltrating cells single and in streams (located more towards perimeter of the tumor were negative). Stromal CT cells positive. 4. normal breast lobular unit cytoplasm 1+, nucleus	Entire specimen staining positive 2-3+ cytoplasm and 2+ nucleus.	1. Invasive carcinoma - negative. 2. Occasional positive cells in small acinar carcinoma areas surrounded by lymphocytic infiltration. Nuclear. 3. normal end units scattered positive cells. Nuclear.	1. ductal areas and solid groups surrounded by lymphocytes and inflammatory cells stained 1+ cytoplasm. No nuclear staining. 2.Undifferentitiated invasive tumor in small groups were very weak staining, lower level of expression. <1+, cytoplasm. 3. Undifferentiated infiltrating cells single and in streams (located more towards perimeter of the tumor) were negative. 4. normal breast lobular unit cytoplasm ≤1+, cytoplasm
	12746-07	1390 G	L Breast N	1. Has some normal - same as #4 above i.e. low intensity staining mostly cytoplasm and also nucleus. 2. Also invasive carcinoma - negative.	tumor and normal cytoplasm 2+	1. Invasive carcinoma - negative. 2. Normal end units- most cells positive, nuclear.	1. Invasive carcinoma - negative. 2. Normal end nits most cells positive, membrane strongest and cytoplasm. 3.Some end units low level of cytoplasmic staining.
2.	12748-09	13443	R breast T	Invasive carcinoma 1+ cytoplasm	Entire specimen - carcinoma staining positive 3+ cytoplasm and 2+ nucleus.	negative	negative
	12748-05	1344 G	R breast N	collagen only - no breast lobule or duct in sample.	no sample	collagen only - no breast lobule or duct in sample.	larger specimen - abundant collagen. Some small end units - exhibit membrane staining. Generally low level of expression.
3.	013365-5		Breast CA	negative. Tumor has multiple areas of poorly defined lobular-like pattern separated by strong collagen deposition.	Within each of lobules variable staining. Some cells negative. Other cells positive cytoplasm 1-2+ and nucleus 1+. Other lobules stronger cytoplasmic staining 2-3+. Some lobules exclusively nuclear 1-2+. Some lobules stroma is staining 1-2+. The staining tends to be positive toward center and negative at perimeter of each lobule.	Positive nuclear staining. Note distribution follows pAkt pattern, with negative staining at in cells perimeter.	negative
	013365 D		Breast NAJ	includes 1. ducts with hyperplasia - negative 2. Area of cystic ducts with duct with in situ - negative. REVIEW	all sites stain cytoplasm 2+ nucleus 2+	Variable: 1. hyperplastic ducts all cells positive. 2. End units scattered positive cells. 3. In situ showed many cells positive, 4. cystic acinar the lining cells wee negative. Nuclear.	Variable, generally <1+: 1. hyperplastic ducts low level staining cytoplasm predominately, rare membrane. 2. End units some few positive cells, cytoplasm, very weak staining. Others negative. 3. Hyperplastic acinar units [positive cytoplasm weak and also membrane staining, moderate. Cystic dilated acini variable negative predominately, some weak positive cytoplasm.
4.	13485 11		breast CA	cytoplasm 1+; nucleus negative	not done	negative	Entire tumor positive. Cytoplasm and membrane. High level of expression 3+
	13485-13		Breast NAJ	not done	ducts and end units expression in cytoplasm 1-2+; nucleus 1+.		negative
5.	13546-07	199K	L breast N	1. ducts negative. Some end units 1+ cytoplasm. Some only individual cells positive. 2. no tumor in specimen.	1. ducts and end units expression in cytoplasm 1-2+; nucleus 1+ 2. no tumor in specimen.	1. ducts and end units positive, most cells, nucleus 1+ 2. no tumor in specimen	1. ducts and end units general negative. However some were positive, low levels of expression, cytoplasm and also membrane ≤1+ 2. Tumor invasive Carcinoma - positive membrane 1-2+
6.	13819-03	1539	L Breast T	negative	ducts and end units expression in cytoplasm 1-2+; nucleus 1+	Almost entirely positive nuclear, also cytoplasm (variable). Some neoplastic cells negative.	Negative. Note: However a few localized ductal elements within tumor showed very weak staining including membrane and cytoplasm.
	13819-05	1539	L Breast N	not done	not done	acinar positive ,nuclear	Negative.
7.	12867-15	1410	L Breast	not done	not done	negative	Most of tumor positive 2+.Some area 1+
	12867-17	1410	L Breast	not done	not done	end units most cells positive. Ducts some cells positive	Variable some end units and ducts negative. Other end units very low level of expression pale staining cytoplasm. Also membrane.
8	13541-04	1499F	L Breast T	not done	not done	negative	1. Most of tumor 60% low level of expression 1+. Some areas negative. 2. normal end unite also positive very weak staining, cytoplasm <1+